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The Macrophage Inhibitory Cytokine Integrates AKT/PKB and MAP Kinase Signaling Pathways in Breast Cancer Cells.

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ABSTRACT

Macrophage inhibitory cytokine 1 (MIC-1), a divergent member of the transforming growth factor beta superfamily, plays a role in progression of number of cancers including breast, gastric, prostate and colorectal carcinomas. Serum MIC-1 levels are elevated in patients with metastatic prostate, breast and colorectal carcinomas. In vitro studies have revealed cell type specific role for MIC-1 in senescence and apoptosis. MIC-1 activates the survival kinase AKT/PKB in neuronal cells. Depending on cell type, it activates or represses MAP kinases ERK1/2. Mechanisms responsible for increased MIC-1 expression in cancers and consequences of MIC-1 overexpression, however, are not known. In this study, we show that AKT/PKB directly regulates the expression of MIC-1 in breast cancer cells. Sequences within –88 to +30 of the MIC-1 promoter are required for AKT-mediated induction of MIC-1. This region of the promoter contains two SP-1 binding sites (SP-1B and SP-1C), which bind to SP-1 and SP-3 proteins. Mutation of SP-1C but not SP-1B reduced AKT-mediated activation of MIC-1. MIC-1 increased basal ERK1 phosphorylation and prolonged estrogen-stimulated ERK1 phosphorylation in MCF-7 breast cancer cells without altering the phosphorylation status of AKT/PKB. Immunohistochemistry with MIC-1 antibody revealed MIC-1 expression within cancer cells of primary breast cancer and in MCF-7 xenografts. Furthermore, a limited analysis of RNA from primary breast cancers revealed overexpression of MIC-1 in tumors compared to normal tissues. These results suggest that AKT/PKB through MIC-1 can regulate ERK1 activity and MIC-1 expression levels may serve as a surrogate marker for AKT activation in tumors.

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INTRODUCTION

The serine/threonine kinase AKT/PKB is a major cell survival kinase for a number of cell types including breast cancer cells [1]. It is activated by growth factors including epidermal growth factor, platelet derived growth factor and heregulin [1]. Constitutive activation of AKT is observed in a number of cancers including breast cancer [2-4]. Constitutively active AKT along with telomerase can immortalize mammary epithelial cells [5]. In transgenic mouse model, constitutively active AKT alone induces hyperplasia and collaborates with ErbB2 to induce mammary carcinogenesis [6,7]. It is also suggested that AKT/PKB phosphorylates Raf kinase in breast cancer cells leading to inhibition of Raf-MEK-ERK signaling and shifting of cellular response from cell cycle arrest to proliferation [8,9].

We recently reported AKT/PKB-mediated activation of estrogen receptor alpha (ERα) and tamoxifen resistance in MCF-7 cells [10]. MCF-7 cells engineered to overexpress constitutively active AKT showed ~5 fold increase in the expression of macrophage inhibitory cytokine (MIC-1). MIC-1 (also known as NAG-1, GDF-15, PDF, PLAB and PTGFB) is a divergent member of transforming growth factor superfamily and is considered as a biomarker for p53 pathway activation [11-13]. Although in vitro studies suggested anti-proliferative and pro-apoptotic functions of MIC-1 in breast, prostate, colon cancers as well as in glioblastomas, analysis of serum samples of patients showed a striking correlation between elevated MIC-1 levels and metastatic progression of colorectal, breast and prostate cancers [14-21]. MIC-1 level is also elevated in the serum of patients with pancreatic ductal adenocarcinoma [22]. MIC-1 increases invasiveness of gastric cancer cells by upregulating the expression of urokinase plasminogen activator [23]. Elevated levels of serum MIC-1 is associated with cardiovascular events whereas lower level of MIC-1 is a predictor for miscarriage in women [24,25]. MIC-1 is also overexpressed in cancer cells undergoing senescence in response to chemotherapeutic treatment [26]. These pleiotropic effects of MIC-1 resemble that of TGFβ, which is considered as a tumor suppressor during early stages of cancer and growth/metastasis enhancer at later stages of cancer [27].

This study was initiated to understand the role of AKT in regulating MIC-1 expression and to delineate the consequences of MIC-1 expression on basal and inducible ERK1/2 and AKT activity in breast cancer cells. We show that AKT directly increases MIC-1 expression in breast cancer cells through a SP-1 binding site. We also show that recombinant MIC-1 increases basal ERK1 phosphorylation and prolongs estrogen-induced ERK1 phosphorylation. These results suggest that MIC-1 serves as an integrator of AKT and ERK pathways in breast cancer cells.

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MATERIALS AND METHODS

Breast cancer cells: MCF-7 and ZR-75-1 breast cancer cells were purchased from American Type Tissue Culture Collection (Manassas, Virginia) and maintained in MEM plus 10% fetal calf serum.

Recombinant plasmids and transient transfection: MIC-1 promoter corresponding to nucleotides – 966 to +30 was amplified from the human genomic DNA by polymerase chain reaction (PCR) and the amplified product was cloned into the chloramphenicol acetyl transferase reporter vector pBL-CAT3+ [28]. MIC-1 promoter sequences were verified by sequencing. Promoter deletion mutants were constructed by PCR. SP-1 mutants of MIC-1 promoter were constructed using single-step mutagenesis kit from Stratagene (Cedar Creek, TX). Cells were harvested 48 h after transfection and CAT assay was performed as described previously (10). Constitutively active AKT (CA-AKT) and kinase dead AKT (KD-AKT) constructs have been described previously [10]. MCF-7 cells overexpressing CA-AKT was generated using the bicistronic retrovirus vector pQXIN (BD Sciences, Palo Alto, CA). All CAT assays were performed three to six times.

Western blot analysis: Cell lysates were prepared in radioimmunoassay buffer and subjected to Western analysis as described previously [10]. Phospho-AKT (S473), AKT, phospho-ERK and ERK antibodies were purchased from Cell Signaling (Beverly, MA). Recombinant heregulin β1 and MIC-1 were purchased from R&D systems (Minneapolis, MN).

Electrophoretic mobility shift assays (EMSA): EMSA was performed using nuclear extracts from MCF7pQXIN and MCF-7CA-AKT cells. Nuclear extract preparation and EMSA conditions have been described previously [29]. In competition assays, nuclear extracts were incubated with unlabelled SP-1 consensus sequence containing oligonucleotides or –88 to +32 region of the MIC-1 (50 fold access) for 10 minutes on ice before addition of labeled –88 to +32 region of the MIC-1 promoter. In supershift assays, nuclear extracts along with probe was incubated with SP-1 or SP-3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 10 minutes before electrophoresis.

Immunohistochemistry: Sections 4 microns in thickness were deparaffinised and hydrated. Antigen retrieval was performed in 140mM citrate buffer pH 6.0 in a decloaking chamber (Biocare) at 125°C for five minutes. The slides were then allowed to cool for 20 minutes at room temperature. The endogenous peroxidase activity was blocked by Peroxo-Block (Zymed) for two minutes. The slides were then incubated with

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2% gelatin for 30 minutes to decrease the non-specific staining followed by blocking endogenous avidin-biotin activity by Avidin-Biotin Blocking kit (Vector Laboratories). The slides were incubated with 1: 100 goat polyclonal MIC1 antibody (R&D systems) for one hour at room temperature. For the subsequent steps, the avidin–biotin–peroxidase method with a Vectastain ABC *Elite* kit (Vector laboratories) was used according to the kit instructions. The stain was visualized using Dako liquid DAB plus substrate chromogen solution and hematoxylin QS (Vector laboratories) counter stain.

Analysis of MIC-1 expression in primary breast cancer by reverse transcription-polymerase reaction (RT-PCR). Total RNA from tumor tissues or the adjoining normal tissue was isolated by guanidinium isothiocyanate method. RT-PCR was performed using the single step RT-PCR kit from Invitrogen. The primers used were 5'-CGCTCCGCGCGTCGCTGGAAG-3' and 5'-GGAGCGACTCCCCGGTGTCGG-3' for MIC-1; 5'-TGGAGAAACTGCTGCCTCAT-3' and 5'-GGAGATGTTGAGCATGTTCA-3' for 36B4.

Statistical Analysis: Data were analyzed using GraphPad software (Graphpad.com). Analysis of variance was employed to determine *p* values between mean measurements. A p value < 0.05 was deemed significant. Error bars on all histograms in this text represent standard deviation between measurements from 3-5 experiments.

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RESULTS

MIC-1 expression is elevated in breast cancer.

We have previously shown elevated expression of MIC-1 in MCF-7 breast cancer cells overexpressing constitutively active AKT. These cells are also resistant to tamoxifen. To determine whether MIC-1 is overexpressed in primary breast cancers, we performed semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) of RNA isolated from primary breast cancers and adjacent normal tissue. MIC-1 transcript levels were elevated in primary breast cancer samples compared to adjoining normal tissues (Fig. 1A). To investigate whether MIC-1 is expressed in cancer cells or stromal cells, we performed immunohistochemistry of primary tumor samples. Only cancer cells express MIC-1 (Fig. 1B).

We used MCF-7 cell derived tumors in nude mice to evaluate whether stromal-cancer cell interaction plays a role in MIC-1 expression. These tumors were obtained from animals with slow-release estrogen pellets. MCF-7 cells form tumors in nude mice only when supplemented with estrogen [30]. MCF-7 cells maintained in vitro do not express MIC-1 [10]. However, MIC-1 was readily detectable in MCF-7 xenografts (Fig. 1C). Thus, it appears that stromal:cancer cell interaction contributes to MIC-1 expression in cancer cells.

AKT directly increases MIC-1 expression. Because our previous studies have shown a role for AKT in MIC-1 expression and AKT is activated in breast cancer due to autocrine and paracrine activity of growth factors, we examined whether AKT directly regulates MIC-1 promoter activity. Previous studies have identified several regulatory elements in the MIC-1 promoter [28]. The basal transcription requires three binding sites for SP-1/SP-3 transcription factors (SP-1A, SP-1B and SP-1C), which are located between –133 to +41 region of the promoter. SP-1B is a perfect SP-1 binding site (GGGCGG) and binds preferentially to SP-1 and SP-3 whereas SP-1C is an imperfect SP-1 binding site (AGGCGG) and binds poorly to SP-1 and SP-3. However, it binds strongly to a truncated SP-3 produced via internal translation initiation [28]. To determine whether AKT utilizes any of these regulatory elements to increase MIC-1 expression, we transfected MCF-7 breast cancer cells and LnCAP prostate cancer cells with MIC-1/CAT reporter with or without constitutively active (CA-AKT) or kinase dead AKT (KD-AKT) expression vector [10]. CA-AKT but not KD-AKT increased MIC-1/CAT expression in both cell types (Fig.2A). A series of deletion mutants were made to localize the region of MIC-1 promoter required for AKT-mediated activation. Although deletions affected basal activity of the promoter, AKT-mediated activation was not significantly affected by deletions. A reporter construct with only –88 to +30 of MIC-1 promoter (-88+30/CAT), which contains SP-1B and SP-1C sites, was induced by CA-AKT (Fig. 2B). Thus, AKT

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responsive element is located within –88 to +30 of MIC-1 promoter, which has been previously reported to be important for basal expression of the gene [28].

SP-1C element is required for AKT-mediated activation of MIC-1.

To determine the requirements of SP-1 motifs in AKT-mediated activation of MIC-1 promoter, we mutated SP-1B (MIC1/SP-1Bmut) or SP-1C (MIC1/SP-1Cmut) sites in the –88+30/CAT reporter. The basal activity of MIC-1/SP-1Bmut was similar to that of –88+30/CAT and AKT increased its activity (Fig. 3). In contrast, MIC-1/SP-1Cmut CAT was not efficiently induced by AKT. These results suggest that AKT increases MIC-1 promoter activity through SP-1C site.

AKT does not alter transcription factor binding to the –88 to +30 region of MIC-1 promoter. To determine whether AKT alters binding of transcription factors to the -88 to +30 region, we first generated MCF-7 cells overexpressing CA-AKT (MCF-7CA-AKT) by retrovirus mediated gene transfer. Nuclear extracts from cells with the vector alone (MCF-7pQXIN) or CA-AKT were used for electrophoretic mobility shift assay (EMSA) with –88 to +30 as a probe. Similar DNA binding pattern was observed in both cell types (Fig. 4A). Intensity of complex I varied from experiments to experiments (Compare Fig. 4A and B). Unlabelled oligonucleotide containing consensus SP-1 binding site reduced the intensity of this complex I suggesting that this complex corresponds to SP-1:DNA complex (Fig. 4B). Interestingly, competition with unlabelled SP-1 consensus oligonucleotide increased binding of a minor slow mobility complex (indicated by asterisk).

We used antibody supershift assays to determine whether SP-1 or SP-3 bind to –88 to +30 region. SP-1 antibody disrupted complex I whereas complex II and III as well as the minor complex (corresponding asterisk in Fig. 4A) were supershifted by SP-3 (Fig. 4B). Taken together, these results indicate that AKT alters the activity but not binding of a transcription factor(s) to the –88 to +30 region of the MIC-1 promoter. SP-1 and SP-3 appears to be the major proteins that bind to this region of the promoter.

MIC-1 increases ERK-1 phosphorylation in MCF-7 and ZR-75-1 cells.

ERK pathways play a distinct role in growth and differentiation of breast cancer cells. For example, transient activation of Raf-MEK-ERK along with activation of AKT pathway by insulin like growth factor leads to proliferation of MCF-7 cells [31,32]. However, prolonged activation of Raf-MEK-ERK cascade leads to growth inhibition of these cells. The PI3 kinase/AKT pathway helps in transient Raf-MEK-ERK activation by downregulating Raf activity through phosphorylation of S^{259} [8,9]. Anti-estrogens such as tamoxifen has been shown to induce proliferation instead of inhibiting the growth of

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MCF-7 cells with elevated basal ERK activity [33]. A recent report suggested that MIC-1 activates PI3 kinase/AKT but represses ERK pathway in neuronal cells [34]. In gastric cancer cells, however, MIC-1 induces ERK pathway [23]. These contrasting cell type-specific effects of MIC-1 on ERK prompted us to investigate the effect of MIC-1 on basal, estrogen and heregulinβ1-inducible ERK and AKT activation in MCF-7 cells. Contrary to neuronal cells, MIC-1 increased basal ERK phosphorylation (Fig. 5A). ERK1 is the major active ERK in MCF-7 cells. Estrogen induced ERK1 in a biphasic manner in the absence of MIC-1 whereas ERK1 activation was continuous in cells treated with both estrogen and MIC-1. MIC-1 had a modest effect on heregulin β1-induced ERK activation (Fig. 5A). We next examined the effect of MIC-1 on AKT activation. MIC-1 had minimum effect on basal or inducible AKT activation.

We determined the effect of MIC-1 on ERK activation in ZR-75-1 cells to ensure that the observed effects are not specific to MCF-7 cells. Unlike in MCF-7 cells, both ERK1 and ERK2 are active in these cells to similar extent (Fig. 5B). Surprisingly, estrogen reduced phospho-ERK2 levels and altered ratio between phospho-ERK1 and phospho-ERK2 in favor of phospho-ERK1. MIC-1 pre-treatment accelerated estrogen-induced shift in phospho-ERK1 and phospho-ERK2 ratio. Neither estrogen nor MIC-1 altered overall levels of ERK1 and ERK2. As with MCF-7 cells, MIC-1 had no effect on phospho-AKT levels. From these results, we conclude that MIC-1 enhances phosphorylation status of ERK protein with a specific effect on ERK1. Also, the effect of MIC-1 on ERK is not negatively regulated by AKT.

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DISCUSSION

In this report, we show a crosstalk between MIC-1, ERK and AKT in breast cancer cells. While AKT increased MIC-1 expression, MIC-1 elevated ERK activation. MIC-1 either alone or in combination with estrogen increased phosphorylation of mostly ERK1. The role of ERK in growth and differentiation of mammary epithelial cells is still not clear. Although initial studies indicated a role for ERK in proliferation of mammary epithelial cells, its activity has been shown to be required for differentiation of breast cancer cells by agents such as TPA [31]. MCF-7 cells with elevated basal ERK activity has been shown to growth-stimulated by tamoxifen [33]. AKT pathway on the other hand is considered a major survival and proliferation pathway for mammary epithelial cells [8,35]. It is suggested that AKT blocks differentiation of mammary epithelial cells by inhibiting ERK [8]. The negative crosstalk between AKT and ERK may be abrogated in cells that overexpress MIC-1 because MIC-1 can induce ERK without reducing AKT and MIC-1 is induced by AKT.

MIC-1 appears to shift the ratio between active ERK1 and ERK2 in favor of more active ERK1 (Fig. 5). Consequences of such a shift are not known. Although ERK1 and ERK2 perform redundant functions, knockout animal studies have revealed unique functions for ERK1 and ERK2. While ERK2 knockout animals show embryonic lethality due to defective mesoderm differentiation, ERK1 knockout animals are viable and have a minor defect in T cell development [36,37]. Although there are no differences in substrate specificity, ERK1 but not ERK2 binds to scaffold proteins such as MP-1, which enhances its enzymatic activity [38]. By altering ratio between ERK1 and ERK2, MIC-1 in combination with estrogen can increase the level of enzymatically active species of ERK. In this regard, we have observed that higher expression of ERK2 than ERK1 in majority of breast cancer cell lines (data not shown), which may limit the effect of MIC-1 to only those cell types that express higher level of ERK1 compared to ERK2.

AKT increased MIC-1 expression in prostate cancer cells also (Fig. 2). This is particularly interesting because AKT activation in prostate cancer is associated with poor clinical outcome [39]. Interestingly, AKT activation in prostate cancer cells leads to reduced ERK activation, which is similar to the effect of MIC-1 on ERK in neuronal cells [34]. MIC-1 overexpression in prostate cancer is correlated with invasiveness and metastasis [40,41]. A recent protein profiling of microdissected prostate tissue revealed overexpression of MIC-1 protein in high-grade prostatic intraepithelial neoplasia and prostate cancer cells of Gleason grade 3 [42]. Additional studies are required whether AKT action in prostate cancers is mediated through induction of MIC-1.

The mechanism by which AKT increases MIC-1 expression is not known. The basal MIC-1 promoter activity is dependent on three SP-1 response elements; SP-1A, SP-B and SP-1C [28]. SP-1B is a perfect SP-1 binding site whereas SP-1A and SP-1C are

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imperfect SP-1 binding sites. SP-1A site is not involved in AKT-mediated activation of MIC-1 because this site is upstream of the minimum region of the promoter required for AKT-mediated activation. Furthermore, a reporter containing –966 to +30 promoter region of MIC-1 with SP-1A mutation was activated by AKT (data not shown). SP-1C is involved in AKT-mediated activation of MIC-1. SP-1 has previously been linked to AKT-mediated activation of FRA-1 [43]. Activation of FRA-1 by AKT involved transcriptional upregulation and increased DNA binding of SP-1 [43]. However, that is not the case with MIC-1 promoter because we did not observe increased SP-1 binding in cells overexpressing CA-AKT. SP-1C binds preferentially to truncated SP-3 [28]. We also observed preferential binding of SP-3 to the MIC-1 promoter region that respond to AKT (Fig. 4B). We believe that AKT increases interaction of SP-3 bound to SP-1C site with transcriptional co-activators or other transcription factors. One such candidate is ERα, which indirectly activates promoters through interaction with SP-1 [44] and is phosphorylated by AKT [10]. In our previous study, we have shown estrogen-dependent increase in MIC-1 in MCF-7 cells overexpressing CA-AKT [10]. In addition, a number of co-regulators are phospho-proteins and some of them may be target of AKT [45,46]. COUP-TF nuclear receptors have previously been shown to increase MIC-1 expression through SP-1 binding sites [28]. However, COUP-TFs are not likely to be the target of AKT because they lack consensus AKT phosphorylation sites and we did not observe synergistic increase in MIC-1 expression upon co-expression of COUP-TFs and AKT (data not shown). Also note that both SP-1 and SP-3 lack consensus AKT phosphorylation sites.

The role of MIC-1 in cancer progression is unclear if not controversial. We observed MIC-1 expression in primary breast cancer and a recent study showed rapid induction of MIC-1 in cancer cells subsequent to neo-adjuvant therapy [47]. MIC-1 has been linked to senescence and apoptosis of cancer cells [18,26]. It is also overexpressed in cancer cells that are sensitive to chemotherapy but not in cancer cells that are resistant to chemotherapy [26]. In contrast to these pro-cell death function, MIC-1 overexpression is linked to progression of gastric cancer [23]. This paradoxical role of MIC-1 in cancer could be related to its ability to induce ERK. Depending on the cell type and duration of induction, ERK can induce cell cycle arrest, senescence, or drug resistance [33,48,49]. Thus, in cancer types in which ERK induces growth arrest or senescence, MIC-1 may play a pro-death role. Similarly, MIC-1 expression may be linked to sensitivity to chemotherapy in cancers where chemotherapy-induced cell death requires ERK activation [50]. Rapidly proliferating cells are more sensitive to chemotherapy compared to quiescent cells. MIC-1 may sensitize certain cancer cells to chemotherapy by increasing their proliferation. In cancers where ERK activation is linked to survival through AP-1 pathway, MIC-1 may play a role in progression of cancer. MIC-1 mediated

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upregulation of urokinase plasminogen activator in gastric cancer cells could be a consequence of AP-1 activation [23]. With respect to breast cancer, we suspect that MIC-1 plays a role in cancer progression, particularly in estrogen receptor alpha (ERα)-positive breast cancers because it induces ERK without reducing AKT. Both ERK and AKT modulate ERα activity [51]. We observed MIC-1 expression in MCF-7 cell-derived tumors in nude mice (Fig. 1C), which suggests a correlation between proliferation and MIC-1 expression. Elevated MIC-1 in serum of patients with metastatic breast cancer may be indicate rate of proliferation of cancer cells at sites of metastasis [21]. Additional immunohistochemical studies with tissues obtained from sites of metastasis are required to test this possibility.

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FIGURE LEGEND

Fig. 1: MIC-1 expression in primary breast cancer. A) MIC-1 transcripts in breast cancer homogenate as well as in adjoining tissues of some tumors were measured by RT-PCR (35 cycles). The integrity and equal quantity of RNA in each reaction were verified by RT-PCR using primers specific to the housekeeping gene 36B4 (20 cycles). B)

Immunohistochemistry reveals expression of MIC-1 in cancer cells but not stromal cells. C) MIC-1 is expressed in tumors derived from MCF-7 cells in nude mice.

Fig. 2: AKT increases MIC-1 expression. A) CA-AKT but not KD-AKT increases MIC-1 promoter/reporter activity in MCF-7 cells. MCF-7 cells were transfected with a CAT reporter containing sequences –966 to +30 of MIC-1 promoter (10 µg) with or without constitutively active (CA-AKT) or kinase dead AKT (KD-AKT). The empty expression vector pCDNA3 (0.5 µg) was included in the control. The transfection efficiency was measured using RSV-β-galactosidase expression vector (2 µg). CAT activity in equal number of β-galactosidase units was measured 48 h after transfection. CA-AKT increased MIC-1 promoter activity in LnCAP cells. B) The AKT response element is located within the –88 to +30 region of MIC-1 promoter. A series of deletion MIC-1 promoter constructs were transfected into MCF-7 cells with or without CA-AKT as above and CAT activity was measured 48 h after transfection. Note that CA-AKT had no effect on residual expression of pBL-CAT3+ reporter, which lacks any enhancer/promoter region as well as pBL-CAT2+ reporter, which contains HSV TK promoter but not enhancer (data not shown).

Fig. 3: SP-1C element is required for AKT-mediated activation of MIC-1. The –88/+30 MIC-1 CAT reporter with mutation in SP-1B (MIC-1/SP-1Bmut) or SP-1C (MIC-1/SP-1Cmut) were co-transfected with either the empty vector pcDNA3 or CA-AKT. CAT activity was measured as in Fig. 2.

Fig. 4: AKT does not alter transcription factor binding to –88 to +30 region of MIC-1 promoter in vitro. A) Electrophoretic mobility shift assay was performed with nuclear extracts from MCF-7pQXIN (control) or MCF-7CA-AKT cells. The –88 to +30 region of MIC-1 promoter was used as probe. Intensity of complex I was reduced by unlabelled oligonulceotide containing consensus SP-1 binding site identical to SP-1B element. An asterisk indicates a minor complex whose intensity increased upon competition with unlabelled SP-1 consensus oligonucleotide. B) Binding of SP-1 and SP-3 to –88 to +30 region of MIC-1 was determined by antibody supershift assays. While SP-1 antibody disrupted complex I, SP-3 antibody supershifted complex II, III and the minor complex. SS= supershift.

Fig. 5: The effect of MIC-1 on ERK and AKT activity in MCF-7 and ZR-75-1 cells. A) MIC-1 increases phospho-ERK1 levels in MCF-7 cells. MCF-7 cells serum starved for 48 h were incubated with recombinant MIC-1 (25 ng/ml) for one h followed estrogen (10^{-10} M) or heregulin β1 (50 ng/ml) for indicated time. Activated ERK and AKT levels

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were measured using phospho-specific ERK and phospho-AKT (S473) antibodies, respectively. The same blot was reprobed with antibodies that measure total ERK or AKT. Note that MIC-1 increased basal phospho-ERK levels as well as caused sustained instead of biphasic ERK activation in estrogen treated cells. MIC-1 had only a modest effect on heregulin β1-induced ERK. B) MIC-1 increases ERK1 phosphorylation in ZR-75-1 cells and modulates estrogen-mediated changes in phospho-ERK1 and phospho-ERK2 ratio.

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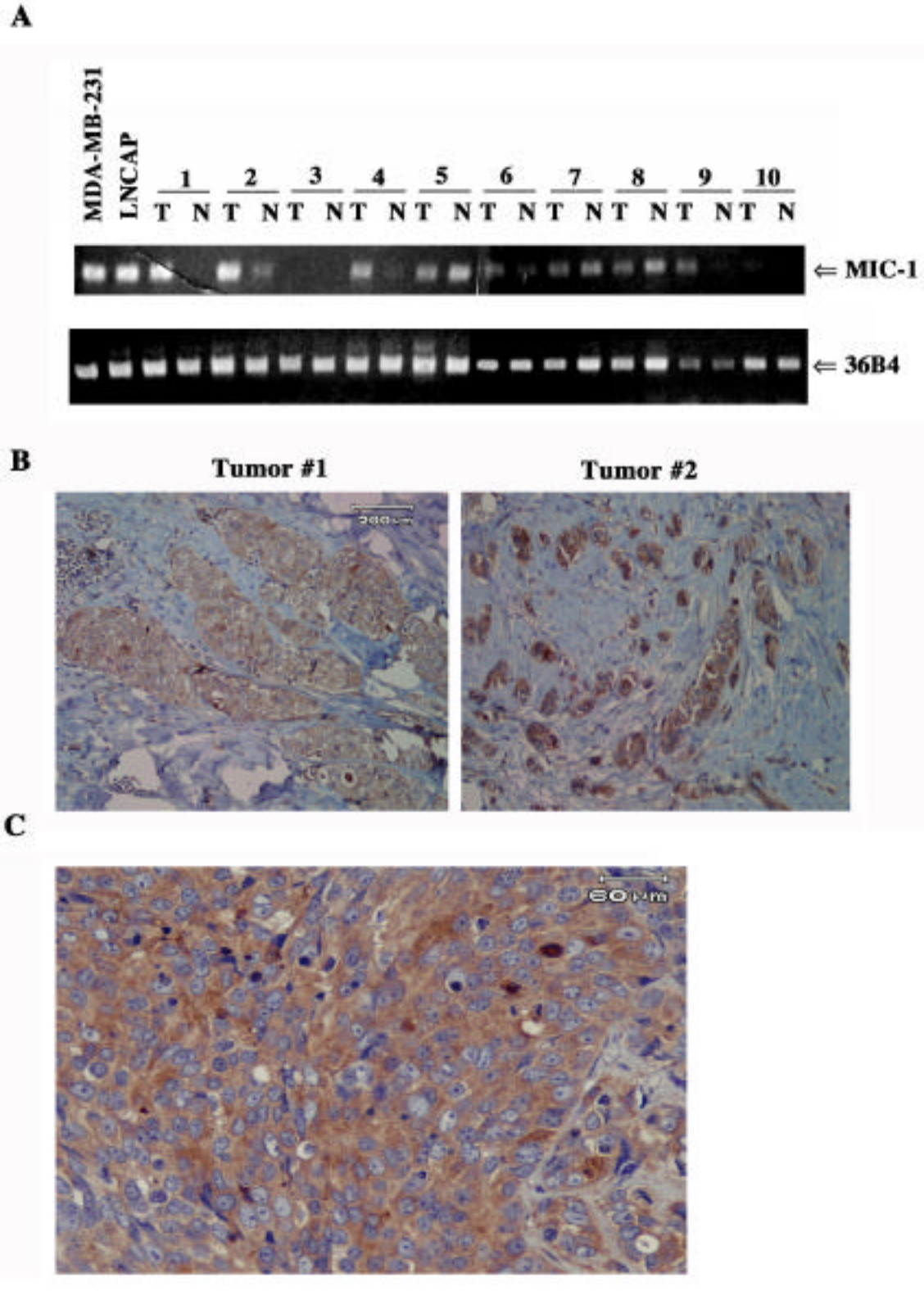

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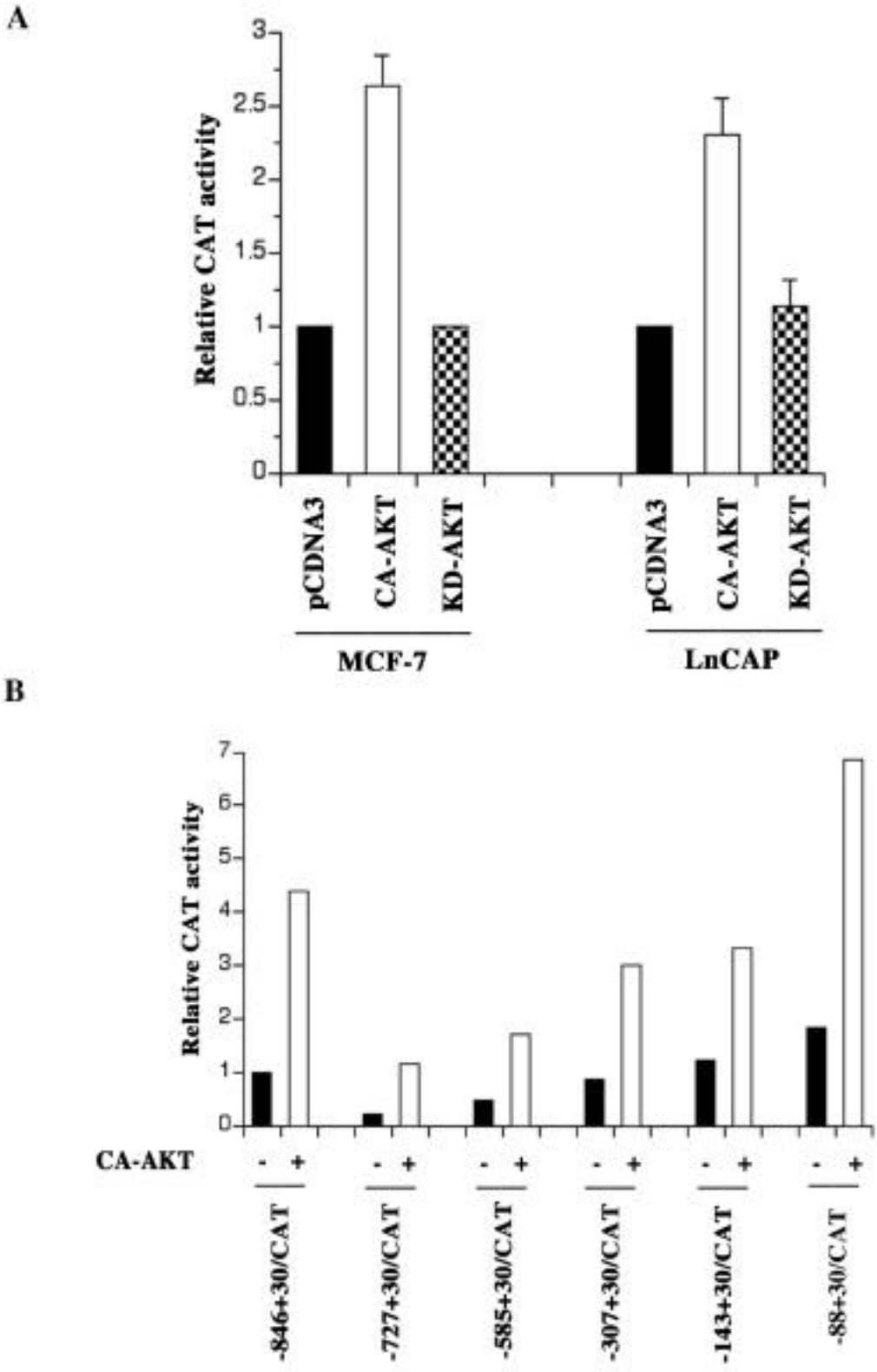

Fig. 2

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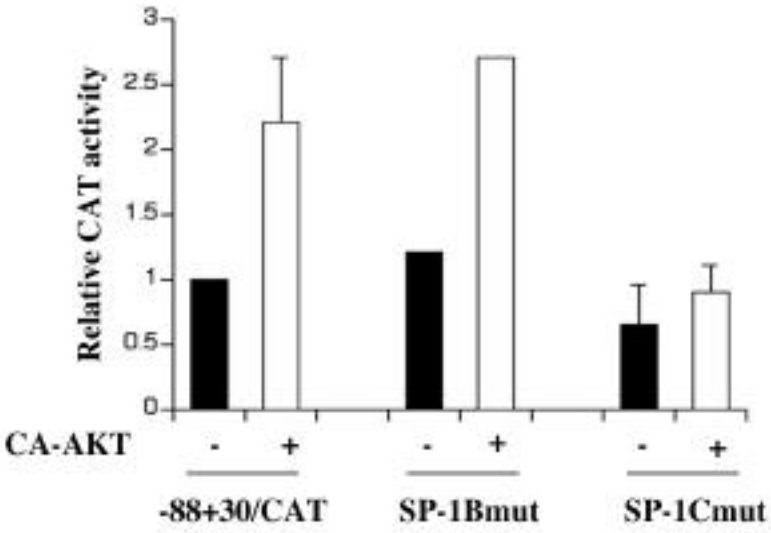

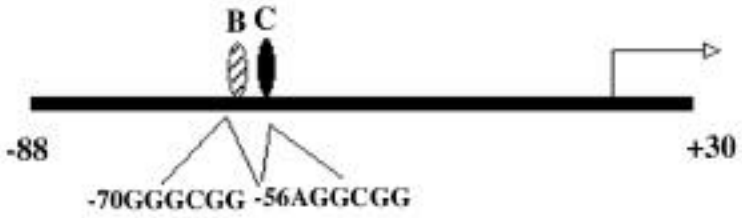

SP-1Bmut GTTCGG

SP-1Cmut ATTCGG

Fig. 3

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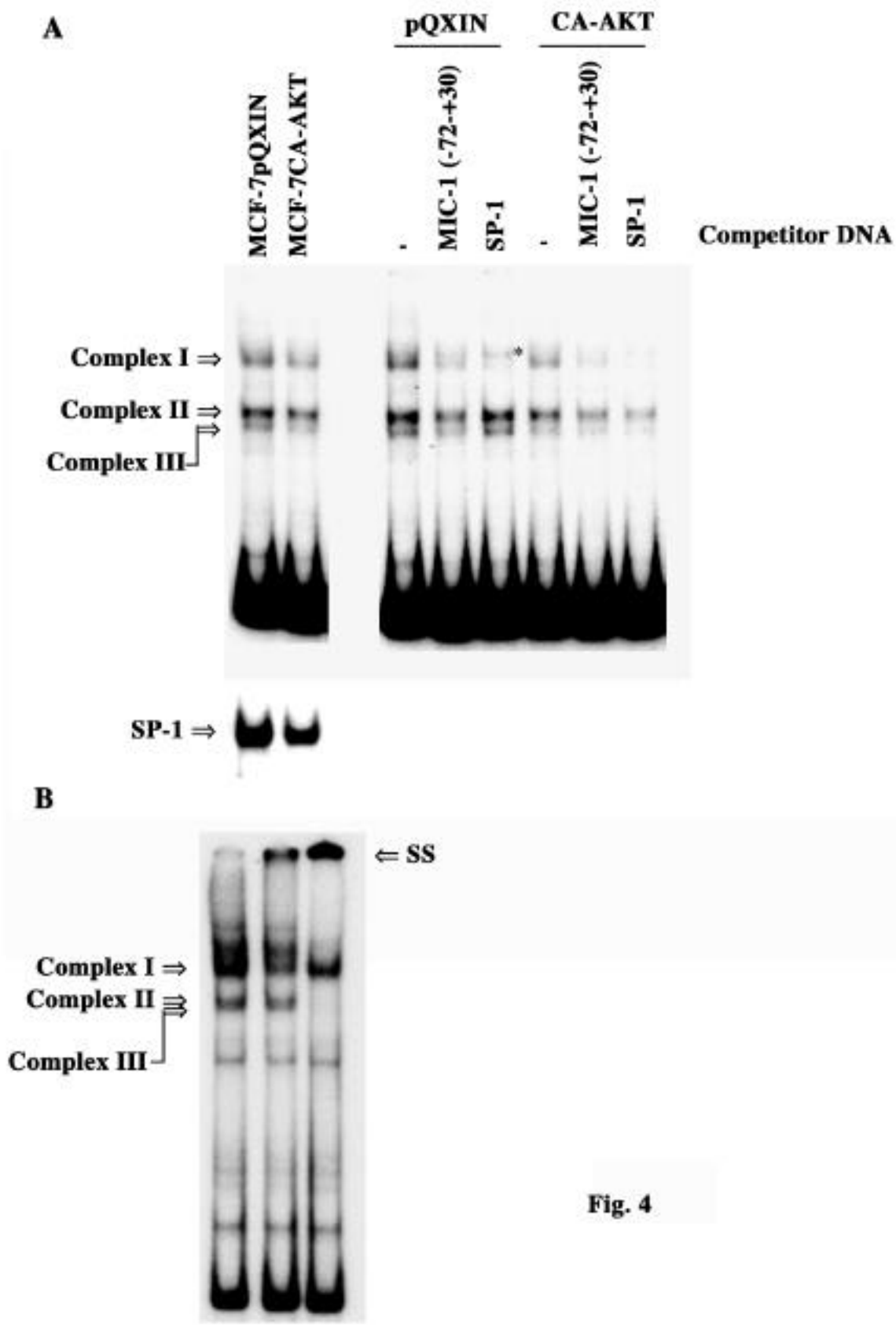

Fig. 4

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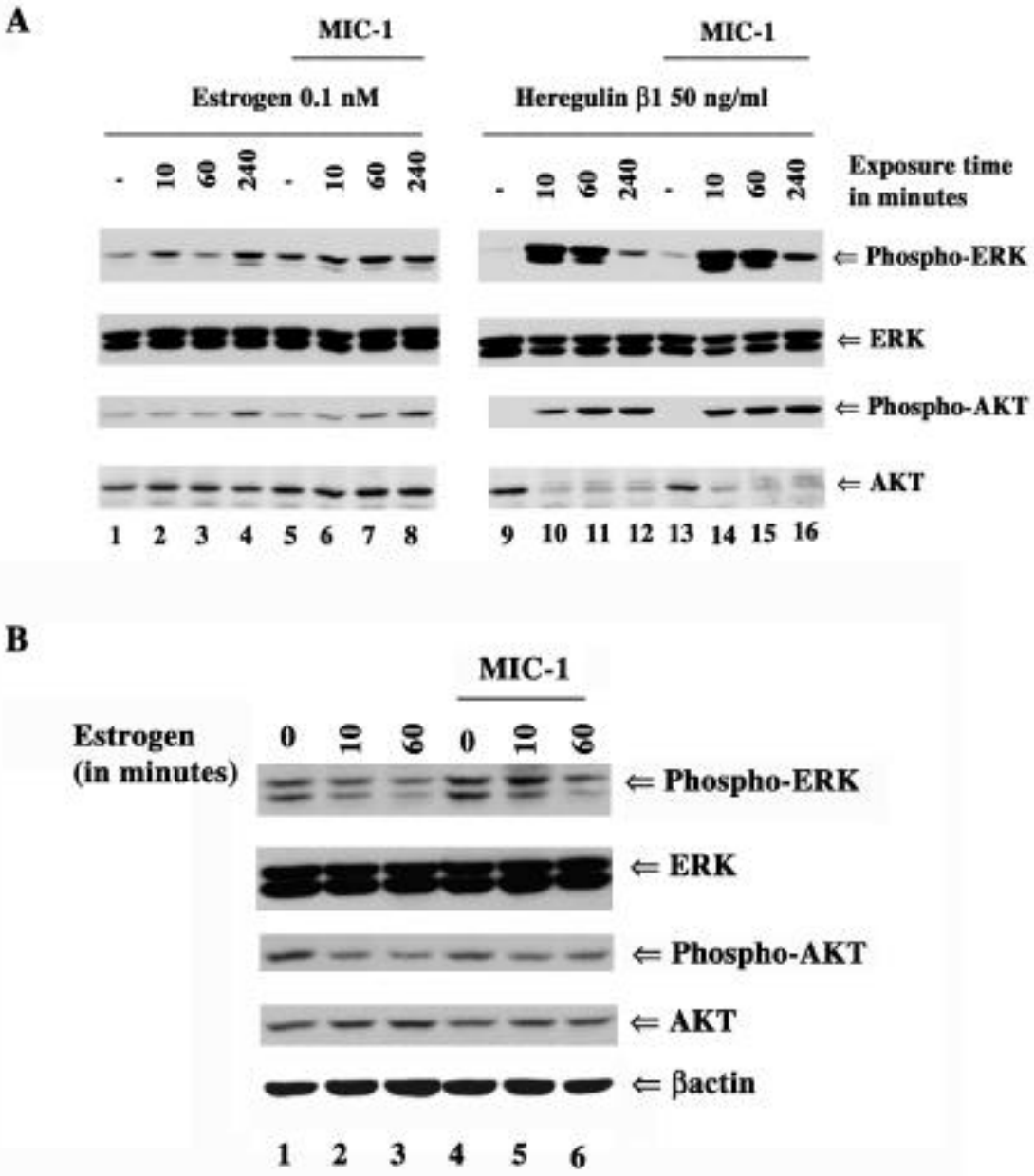

Fig. 5

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